Zein-polycaprolactone electrospun matrices for localised controlled delivery of tetracycline

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Abstract We report the controlled release of the antibiotic tetracycline (Tet) from triple-layered electrospun matrices consisting of zein or a zein/PCL blend, where the drug was loaded in the central layer with the outer two layers acting as diffusion barriers. These fibrous matrices successfully encapsulated Tet and efficiently inhibited the growth of a clinical isolate, the meticillin-resistant *S. aureus* strain MRSA252, as demonstrated in a modified Kirby-Bauer disc assay over five days. Whilst untreated zein fibres are unstable in an aqueous environment, rapidly shrinking due to plasticization and film formation, blending zein with PCL stabilized the electrospun matrices and prevented them from shrinking. These triple layer formulations displayed sustained antibiotic release and provide a proof of concept for zein-based polymeric matrices as wound dressings to treat or prevent bacterial infection. This is the first demonstration of the controlled release of a clinically-used antibiotic from electrospun zein-based matrices.

Keywords Antibiotic; Drug delivery; Electrospinning; Raman microscopy; Sustained release; Wound dressing

Introduction

Electrospinning is a widely utilized technique for generating nano- and microfibres from a wide range of materials, including polysaccharides, proteins and synthetic polymers, alone or in blends [1,2]. A variety of fibre and matrix architectures can be achieved depending on how the fibres are spun and collected. For example, coaxial electrospinning [2,3] can generate fibres with a core and sheath feed consisting of the same or different polymers, often with a drug encapsulated in the centre of the fibres [4]. Modifications in the collection and processing of fibres can result in aligned fibres, tubes and matrices with macropores in addition to the randomly aligned fibres achieved with a stationary collector plate. The procedure is versatile, economical, simple to perform and allows the generation of electrospun structures with a number of desirable properties leading to many potential applications, e.g. packaging, filtration, drug releasing matrices, tissue engineering scaffolds and wound dressings [5-9]. From the perspective of translational medicine, the main advantage of electrospun matrices is that biocompatible nanofibres can mimic the structure and morphology of the extracellular matrix (ECM), resulting in a three-dimensional scaffold that replicates the cell-matrix interactions experienced by cells in vivo. By incorporating drugs or growth factors in electrospun matrices, bioactive implants have been developed for a range of translational applications in different tissues and disease states [9-12]. One such approach is the encapsulation of antibiotics in electrospun fibres [13,14] to treat infection, e.g. in burns patients or those with non-healing ulcers, or to prevent infection following invasive surgical procedures such as joint replacement [15]. This localized delivery from drug-loaded fibres aims to ensure that the affected tissue receives an efficacious dose of antibiotic while the patient avoids the off-target effects that often accompany a systemically-delivered drug.

For such long-term, sustained drug delivery applications, the majority of electrospun matrices described to date consist of a drug or drugs dissolved in a polymer solution, which is then processed to form a blended drug/polymer mat. Sustained drug release necessitates the use of hydrophobic polymers, preventing immediate hydration of the matrix, solubilisation of the drug and diffusion from the fibres. Drug release is controlled by diffusion and the long-term surface and bulk erosion of the matrix, with one erosion mechanism usually dominating. Thus, as the polymer degrades, the drug is released. However, as electrospun matrices are comparable to the ECM and have a very high surface area to volume ratio, there is often a

significant burst release of drug that is on or near the surface of the fibres followed by a sustained release of the remainder of drug, encapsulated deeper within the polymer. To minimize burst release, drugs can be restricted to the fibre core by coaxial electrospinning, or rate-controlling layers can be incorporated to act as a physicochemical barrier to drug diffusion.

There is a broad interest in developing electrospun matrices from natural products and these, to date, include a range of polysaccharides (e.g. alginate, chitosan, and cellulose) [16] and proteins (e.g. collagen, gelatin, laminin, silk fibroin) [10] from a variety of different plant, algal and animal species. However, the majority of these biopolymers are hydrophilic and this property makes them unsuitable for controlled drug release applications due to rapid hydration and drug release. Recently, there has been a renewed interest in the use of zein, the main prolamin storage protein in maize, due to its renewable source, biodegradability, biocompatibility and resistance to microbial degradation [1,17,18]. Zein is also hydrophobic, containing a significant proportion of non-polar amino acids, offering the possibility of sustained release of drugs encapsulated within electrospun zein matrices. Zein has been evaluated for its potential use in applications, including packaging, tissue engineering and drug delivery, either alone or in blends with other materials. For example, films have been employed to demonstrate the biocompatibility of zein with several cell types [5,17,18], and zein-based 3D bulk scaffolds have been postulated as potential scaffolds for bone tissue engineering [7,19-22]. Electrospun zein matrices [15,23] have been reported to be suitable tissue engineering, particularly for skin wound healing applications either alone [16,24] or blended with collagen [25].

For drug delivery applications, zein-based microparticles have been developed for the controlled release of antiparasitic [26], antibacterial [27,28], and anti-inflammatory [29] drugs. Electrospun zein-containing matrices have also been developed for controlled delivery of NSAIDs [30,31], antioxidants [32,33] and the antibiotic berberine [25], while zein/chitosan nanofibres have been shown to be biocidal due to chitosan's known antibacterial properties [34]. However, despite good drug loading and demonstrable controlled release from electrospun zein-based matrices, the time taken for 100% release remains relatively short. For example, the total release of various NSAIDs reported in the literature ranges from 8-12 hours, despite modifications to the electrospinning process aimed to improve the release profile [30,35,36].

In this study, our aim was to develop an electrospun zein-based matrix for the encapsulation and sustained release of a model antibiotic, tetracycline (Tet). This would find

medical applications as a biocompatible wound dressing, but would, ultimately, also be potentially applicable in vivo as an implant for the prevention of infection following invasive procedures or as a tissue engineering scaffold. We report the development of single-layer matrices and, in an effort to reduce burst release, triple-layer (3L) formulations. In the latter, the outer layers are drug-free and designed to act as a diffusion barrier. To our knowledge, these are the first triple-layer, zein-based matrices and demonstrate the first reported extended release of a clinically-used antibiotic from such electrospun zein-based formulations.

Materials and Methods

Materials

Zein was purchased from Acros Organics. Müller-Hinton (MH) agar and Tryptone Soya Broth (TSB) were purchased from Oxoid. The bacterial strain used in this study was *Staphylococcus aureus* MRSA252 [37], kindly provided by Dr Albert Bolhuis, University of Bath. Poly-ε-caprolactone (PCL; Mn 70,000 to 90,000), tetracycline (Tet) HCl and all other chemicals and solvents were purchased from Sigma Aldrich. Gene frames (1x1 cm) and plastic coverslips were purchased from Fisher Scientific.

Polymer solutions

Zein solution was prepared at 30% (w/v) in a 1:1 (v/v) mixture of 2,2,2-trifluoroethanol (TFE):dichloromethane (DCM). Tet HCl was dissolved in TFE at 5% (w/w) with respect to the protein prior to mixing with DCM. For blended matrices of zein and PCL, zein was dissolved at 20% or 25% (w/v) and PCL at 10% or 5% (w/v) in 1:1 (v/v) TFE:DCM, resulting in solutions with a total polymer concentration of 30% (w/v). Tet HCl was again incorporated at 5% relative to the polymer mass.

Electrospinning

The polymer solution was loaded into a glass syringe and electrospun at 18 kV with a flow rate of 0.75 mL/h and the distance between the tip of the needle and the collector set at 13 cm. Flow rate was controlled by a syringe infusion pump (Cole Parmer). The electrospun

matrices were collected on two parallel metal electrodes covered with aluminium foil. To fabricate layered mats, each polymer solution was electrospun using a fixed volume for each layer (1 mL for the outer layers and 0.5 mL for the inner layer) in a layer-by-layer manner. Five matrices were produced: single-layer zein, single-layer zein/PCL (25:5), single-layer zein/PCL (20:10), triple-layered (3L) zein and 3L zein/PCL (20:10). In single-layer matrices, Tet HCl was electrospun at 5% (w/w). In the 3L matrices, Tet HCl was incorporated only in the middle layer at 5% (w/w), with the two outer layers being drug-free. Three replicates of each formulation were fabricated.

Assessment of matrix stability

To investigate the ability of the polymeric fibrous matrices to maintain their morphology when exposed to an aqueous environment, three different single-layer mats were examined: zein, zein/PCL (25:5), and zein/PCL (20:10), all containing Tet HCl. Matrices were punched into 9 mm discs, imaged with a digital camera and then incubated in phosphate-buffered saline (PBS; pH 7.4) at 37 °C for 7 days. Discs were then removed from the buffer, imaged while still wet and then allowed to dry for 3 days at 20 °C before being imaged again. The maximum and minimum diameters of each disc were determined using ImageJ (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/) and the mean diameters compared to that of the discs prior to wetting with PBS buffer. Triplicate samples were examined for each formulation and the experiment was performed three times with independently electrospun mats.

Morphology of electrospun matrices

The surface morphology of electrospun matrices was observed by scanning electron microscopy (SEM; JEOL JSM-6480LV) before and after exposure to PBS at 37 °C. The matrices were cut into small cm^2 sized pieces, sputter-coated with gold (Edwards Sputter Coater 5150B) and then analysed by SEM with an accelerating voltage from 10 kV. The mean fibre diameter was determined by randomly selecting 60 fibres from three separate images and measuring their diameters using ImageJ software.

Raman microscopy

Raman spectra were obtained using a Renishaw inVia Imaging Microscope with a near-infrared diode laser (785 nm) as the excitation source, using approximately 32 mW of power at the sample surface and a 1,200 lines/mm grating. Streamline maps of the sample were obtained at 20 μm line focus with an exposure time of 30 s/line. Irradiation was uniform across the window 900-1,900 cm^{-1}.

Tetracycline hydrochloride encapsulation efficiency

To determine the encapsulation efficiency of the electrospun matrices, the mats were cut into small discs (~6 mm diameter), weighed and then dissolved in methanol:DCM (1:1 v/v, 10 mL). The UV absorbance was measured at λ = 360 nm (subtracting for zein absorbance at this wavelength) and the amount of Tet HCl in the fibres was then calculated using a Tet HCl calibration curve and subsequently compared to the theoretical value (5%). Triplicate samples were examined for each formulation and the experiment was performed three times with independently electrospun mats.

In vitro drug release studies

The electrospun matrices were cut into 1.2 cm squares and, to minimise the effect of drug release from the edges, the samples were adhered to a plastic coverslip using a gene frame to give an available release surface of 1 cm^2. Each sample was placed in PBS buffer (5 mL; pH = 7.4) in a plastic vial and incubated at 37 °C. At specific time intervals, the buffer was replaced and the withdrawn sample assayed by UV spectroscopy at λ = 360 nm to determine Tet release against a standard curve. The cumulative percentage release at each time point was determined by comparing the actual mass release with the mass of Tet in each sample, calculated using the encapsulation efficiency data for each individual mat. Triplicate samples were examined for each formulation and the experiment was performed three times with independently electrospun mats.

Antibacterial efficacy of Tet HCl-loaded nanofibrous layered matrices

Tet HCl-sensitive bacteria, *S. aureus* MRSA252 [37], were used to investigate the efficacy of Tet released from the zein-based electrospun matrices with a modified Kirby-Bauer test [13,14,38]. *S. aureus* suspension was incubated for 16 h in TSB and subsequently diluted

with sterile TSB containing 0.5% glucose (w/v) and 3% NaCl (w/v) to obtain an absorbance value of ~0.035 at 600 nm. Then, 100 µL of the diluted bacteria solution was streaked onto the surface of a MH agar plate (prepared previously according to the manufacturer's instructions) followed by placing the electrospun matrices on the plates. The matrices were cut into discs, each containing 270 µg Tet HCl, with a filter paper disc loaded with 270 µg Tet HCl (applied as a methanolic solution) as a positive control. Three replicates of one formulation and one Tet HCl-loaded filter paper disc were placed on each MH agar plate and incubated at 37 °C for 24 h. After this time, the inhibition zones around the discs were measured and the discs were then transferred to new MH agar plates pre-streaked with fresh bacteria. Following incubation for a further 24 h at 37 °C, the new inhibition zones were determined and this procedure was repeated for a total of five days. The average inhibition zones, determined by measuring the diameter of the zones of inhibition and subtracting the diameter of the matrix disc, were expressed as the mean ± standard deviation (n=3). Student's t-tests were performed using Microsoft Excel to determine any statistically significant differences between the formulations. P<0.05 was considered to be significant.

Results and Discussion

Effect of aqueous environment on electrospun zein and zein/PCL matrices

Initial attempts at electrospinning zein using aqueous alcohol, neat TFE or acetic acid produced inconsistent results with either a complete lack of fibres or significant beading (data not shown). However, electrospinning zein at 30% (w/v) in a 1:1 (v/v) mixture resulted in the successful and reproducible production of matrices with continuous, non-beaded fibres. When discs of electrospun zein matrices were placed in aqueous buffer at 37 °C for 7 days, we observed a drastic reduction in their size from an initial diameter of 9 mm to approximately 3 mm (Fig. 1a), with this shrinkage effect visibly noticeable in <30 min. Even when discs were placed in contact with agar plates, this deformation was marked (data not shown). It is well established that electrospun protein-based fibres lose their integrity in an aqueous environment [39,40]. Hydrophilic proteins such as gelatin and collagen absorb water, swell and dissolve. Zein fibres are also unstable in water, but due to their hydrophobic nature, this instability occurs by a different mechanism. Water has a plasticisation effect on zein [41], with exposure causing a fibrous electrospun matrix to become film-like [35,41-44].

For application of pure, unblended zein fibres as a wound dressing, this shrinkage would be detrimental, as complete coverage of the damaged tissue needs to be maintained. We therefore designed means of overcoming this phenomenon. Numerous methods have been reported in the literature to stabilize protein matrices of various morphologies from particles to sponges to fibres. These methods predominantly rely on chemical crosslinking using a variety of species including genipin [45], glutaraldehyde [46] and carbodiimides [47], although, depending on the protein, other techniques such as autoclaving may be effective [48]. Zein has been successfully crosslinked using hexamethylene diisocyanate [49], while Jiang and co-workers have reported successful stabilization using citric acid either with NaOH catalysis [35] or by heating at 150 °C [43]. However, for drug encapsulation, none of these crosslinking and stabilization methods is suitable due to the possibility of chemical or physical alteration/degradation of the drug molecule, potentially rendering it inactive or even toxic.

An alternative strategy for the stabilization of protein-containing matrices is to blend the protein with a water-stable polymer, and this is established in the literature, in particular for tissue engineering scaffolds [39,40,50]. Therefore, to prevent the drastic shrinkage observed when electrospun zein discs were exposed to water, we investigated blends of zein and PCL, an FDA-approved biocompatible and biodegradable polymer that has been used in areas such as controlled drug delivery and tissue engineering [51]. When solutions of zein and PCL were electrospun at ratios of 25:5 and 20:10 (w/v), using the same conditions as those employed for pure zein, fibrous matrices were successfully prepared. Discs of these matrices were subsequently exposed to PBS for 7 days at 37 °C and no shrinkage was observed (Fig. 1a, b), showing that blending zein with PCL stabilized the protein as intended.

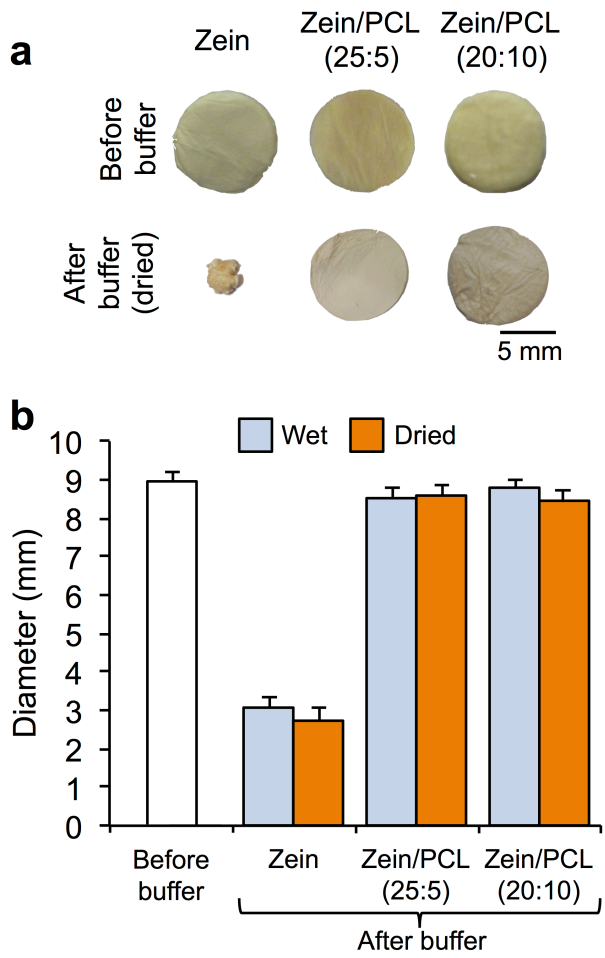

Fig. 1 a Representative images of electrospun single layer zein and zein/PCL matrices prior to exposure to aqueous buffer (initial diameter = 9 mm) and following immersion in PBS for 7 days at 37 °C. Images of immersed discs were obtained following 3 days drying at 20 °C. Scale bar = 5 mm. **b** Mean disc diameter from all formulations prior to buffer exposure in comparison to disc diameters measured immediately after retrieval from buffer (wet) and following 3 days drying at 20 °C (dried). The *error bars* represent the standard deviation of the mean (n=3 in triplicate)

Matrix Morphology

The morphology of the electrospun matrices was examined by scanning electron microscopy before and after exposure to PBS (Fig. 2). SEM revealed the successful formation of smooth, continuous fibres with no evidence of beading or electrospraying. The diameters of as-spun zein fibres, both single-layer and 3L (Fig. 2a, c) were visually considerably smaller than those of the zein/PCL blends (Fig. 2e, g, i) and, as expected, following incubation in aqueous buffer, the zein matrices lost their fibrous structure and formed films (Fig. 2b, d), confirming the observations of the shrinkage experiments shown in Figure 1. The presence of PCL

within the blended electrospun fibres had a dramatic effect on matrix stability, with no apparent differences between the zein/PCL fibres before and after immersion in buffer (Fig. 1f, h, j).

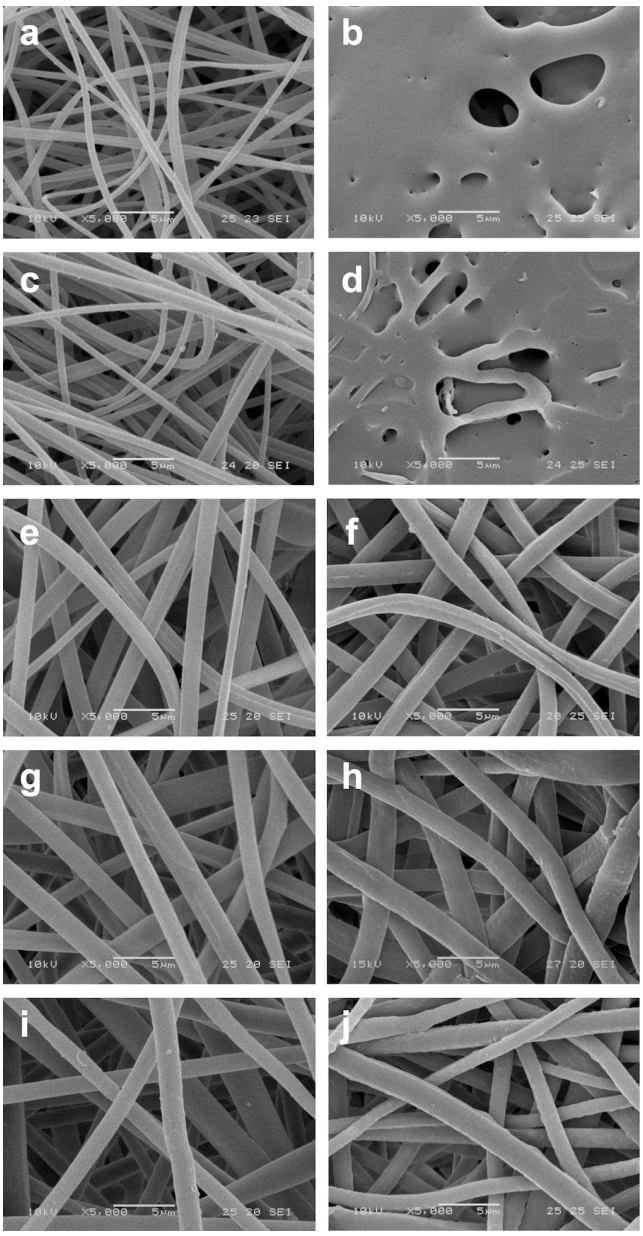

Fig. 2 Representative SEM images of (**a**, **b**) electrospun zein; (**c**, **d**) zein 3L; (**e**, **f**) zein/PCL (25:5), (**g**, **h**) zein/PCL (20:10) and (**i**, **j**) zein/PCL (20:10) 3L matrices before (**a**, **c**, **e**, **g, i**) and after (**b**, **d**, **f**, **h, j**) immersion in PBS at 37 °C. Matrices were immersed in buffer for 7 (**b**, **f, h**), 14 (**j**) or 32 days (**d**). Scale bars = 5 µm

Average fibre diameters were determined from the SEM images and are shown in Table 1. The average diameter of fibres in a single-layer of zein was 0.67±0.19 µm in comparison to 0.99±0.36 µm for the zein 3L matrix. This increased fibre diameter in the 3L

formulation may be explained by two factors. Firstly, the SEM images were of the top surface of the electrospun matrices meaning that, for the single-layer matrix, the observed fibres contained Tet HCl while, for the 3L matrix, the visible fibres were drug-free. As a charged species, Tet HCl increases the conductivity of the electrospinning solution with a resultant decrease in fibre diameter, potentially explaining the smaller diameter of single-layer zein fibres. Alternatively, it has been previously shown that as fibres are deposited during the electrospinning process, the charge build-up on the matrix surface decreases fibre stretching, thus increasing diameter [52]. This phenomenon could explain the greater diameter of the observed zein 3L fibres. An investigation of the dimensions of internal fibres could have helped determine the relationship between spinning conditions and fibre diameter. Unfortunately, attempts to freeze-fracture 3L matrices proved unsuccessful.

Electrospun zein/PCL fibres were thicker than zein fibres and had a greater variability, with diameters of 1.96±0.69 μm for single-layer zein/PCL (25:5), 1.69±0.60 μm for single-layer zein/PCL (20:10) and 1.51±0.65 μm for zein/PCL (20:10) 3L (Table 1). This increase in diameter may be due to changes in the conductivity and viscosity of the electrospinning solution on addition of PCL. However, amongst the blended matrices themselves, there were no apparent trends in fibre thickness. If a relationship exists between relative PCL concentration or the effect of layering on fibre diameter, it may be masked by the variation that was observed in fibre thicknesses on individual matrices. When the effect of immersion in buffer on fibre diameter was examined, there was again no apparent difference between the individual formulations and no changes compared to the as-spun diameters, demonstrating the stability of these blends in an aqueous environment (Table 1).

	Mean fibre diameter (μm)	
Matrix	**Before**	**After**
Zein	0.67 ± 0.19	Film[a]
Zein 3L	0.99 ± 0.36	Film[c]
Zein/PCL (25:5)	1.96 ± 0.69	1.34 ± 0.45[a]
Zein/PCL (20:10)	1.69 ± 0.60	1.92 ± 0.43[a]
Zein/PCL (20:10) 3L	1.51 ± 0.65	1.30 ± 0.59[b]

Table 1. Diameters of electrospun zein and zein/PCL fibres before and after immersion in PBS at 37 °C. Diameters of fibres on the top surface of each matrix were determined from SEM images and are expressed as the mean ± standard deviation of 60 fibres per formulation. Matrices were exposed to PBS for [a]7, [b]14 or [c]32 days

Determination of drug and polymer distribution by Raman microscopy

For eventual applications in wound dressings, it is important that the components of the electrospun matrices, especially the drug, are homogeneously distributed to ensure consistent release. To assess this, Raman spectra were obtained individually for zein, PCL and Tet and, using this information, Raman microscopy was used to image the individual components of electrospun zein/PCL (20:10) with 5% (w/w) Tet. Uniform distribution of all three species was observed (Fig. 3).

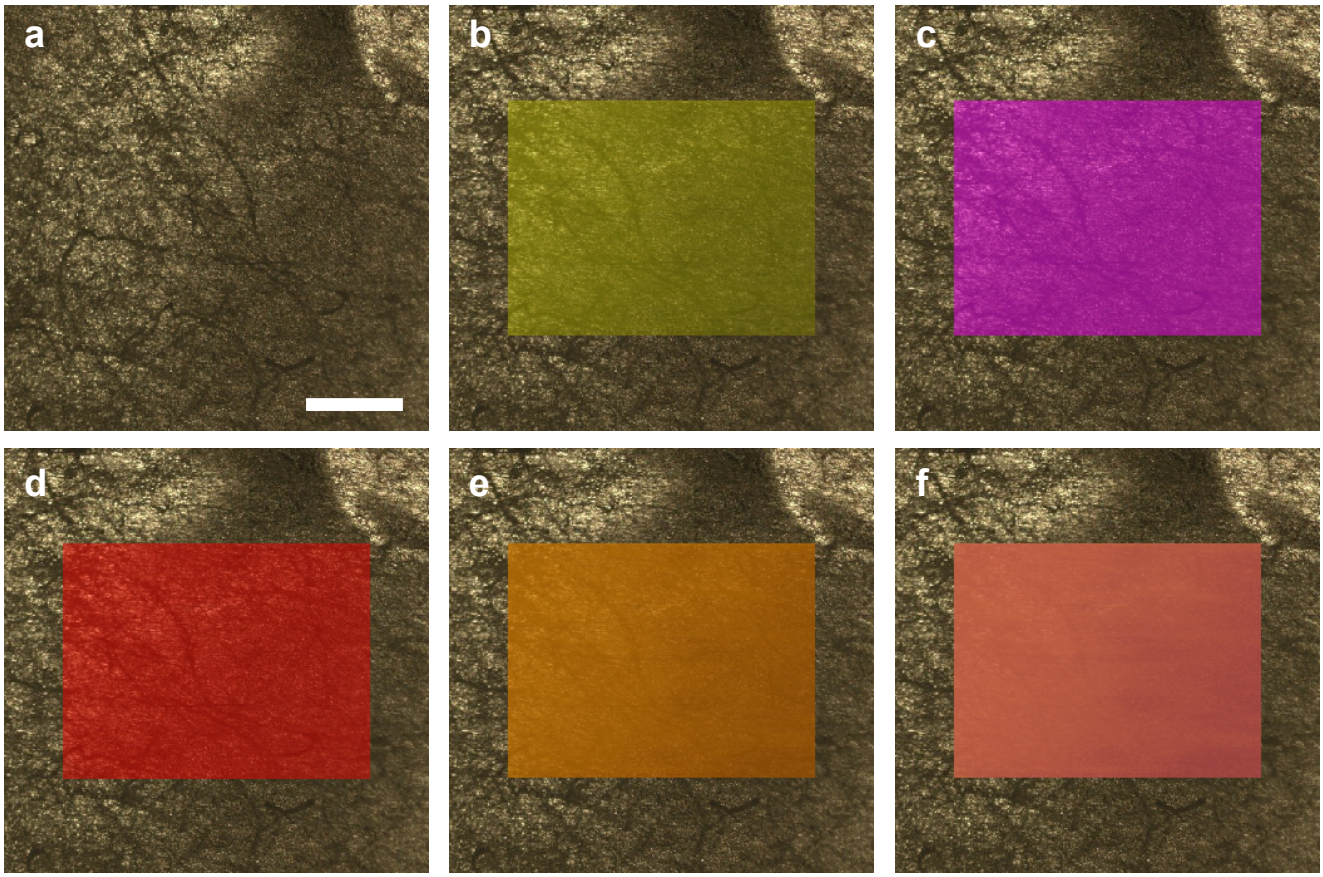

Fig. 3 Raman microscopy of an electrospun zein/PCL (20:10) blend with 5% (w/w) Tet. (**a**) White light; (**b**) zein; (**c**) PCL; (**d**) Tet; (**e**) zein + Tet overlay and (**f**) zein + PCL +Tet overlay. Scale bar = 1 mm.

In vitro release of tetracycline zein and zein/PCL matrices

Prior to release studies, the encapsulation efficiency of each matrix formulation was determined. Within experimental error, encapsulation was quantitative for all but the zein/PCL (20:10) 3L samples, which had an encapsulation efficiency of 71±11%. The release

profiles of Tet from the electrospun zein and zein/PCL matrices into PBS at 37 °C are shown in Figure 4. The single-layer zein matrix exhibited a burst release of ~65% of the drug within the first 3 h (Fig. 4a), followed by a sustained drug release up to ~83% at 10 days, after which the release plateaued (Fig. 4b). Zein 3L proved to be a more controlled formulation than the single-layer equivalent, with an initial burst of ~45% of the Tet in the first 3 h (Fig. 4a) followed by a sustained release which continued until the experiment was terminated (27 days), after which ~70% of the encapsulated drug had been released (Fig. 4b). An extension of the sustained release capacity of zein would be expected by the addition of drug-free, rate-controlling layers that sandwich the central, Tet-containing matrix. However, this effect is likely to have been exacerbated by the transition of the fibrous zein matrices to film-like structures (Fig. 2b, d), with the outer layers of the 3L structure presenting more of a physical barrier than if the structure had remained fibrous. The shift from fibre to film occurred rapidly (<30 min), so this effect dominated the release kinetics, resulting in lengthy, sustained Tet release from the zein 3L matrix.

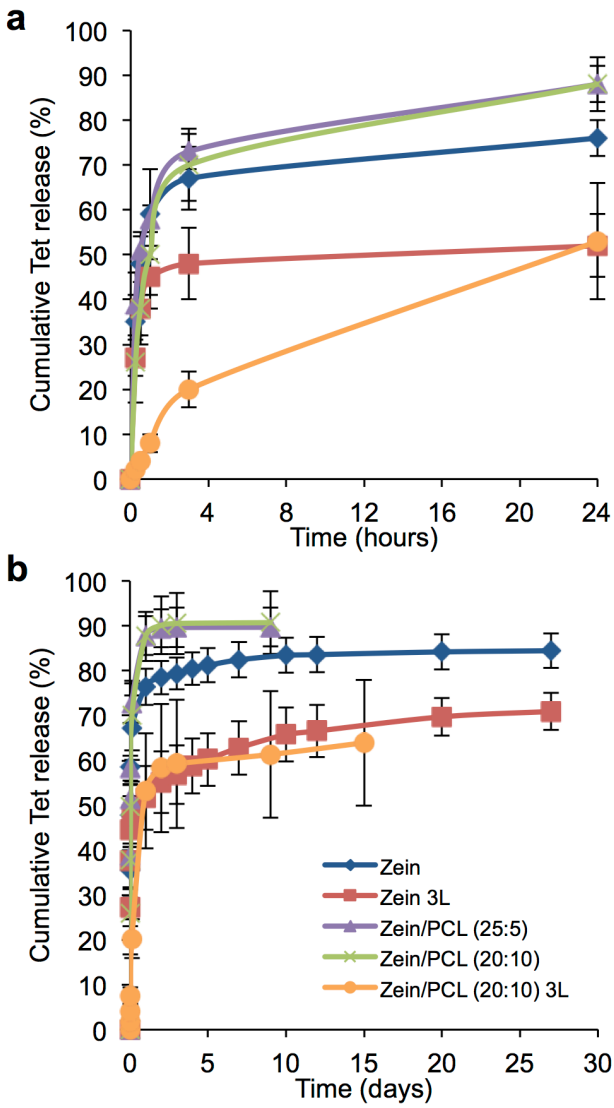

Fig. 4 Release profiles of Tet from electrospun zein and zein/PCL matrices into PBS at 37 °C. **a** First 24 hours and **b** longer term release**.** The *error bars* represent the standard deviation of the mean (*n*=3 in triplicate)

When the single-layer zein/PCL matrices (25:5 and 20:10) were examined, they exhibited near-identical Tet release profiles, with a burst of ~70% of the encapsulated drug within the first 3 h (Fig. 4a), followed by a more sustained release of ~20% of the drug over the next 1-2 days before reaching a plateau (Fig. 4b). In comparison with the single-layer zein matrix, these formulations exhibited a greater burst release and less sustained release. This is possibly due to the chemical nature of the two components of the blended formulations. Although predominantly hydrophobic, zein, as a protein, is amphiphilic, and it would be expected that the encapsulated Tet would interact with the more polar amino acid residues. PCL, however, is hydrophobic and does not possess amphiphilic character, hence blending it with zein is likely to reduce the affinity of the Tet for the matrix and accelerate its release.

Due to their high similarity, only the zein/PCL (20:10) 3L formulation was chosen as it performed marginally better than 25:5 in stability testing and would offer greater mechanical properties for any eventual medical applications. The sharp burst release seen in the other matrices was absent from the zein/PCL (20:10) 3L formulation, with a gradual release of ~50% of the encapsulated Tet over the first 24 h (Fig. 4a). This demonstrates that, in comparison to the single-layer equivalent, the Tet-free outer layers in zein/PCL (20:10) 3L successfully control the release of Tet, acting as a hydrophobic barrier to water ingress and subsequent drug diffusion from the inside of the matrix. This control of drug release continued after 24 h, with a sustained release of a further ~15% of the encapsulated Tet over the next 14 days (Fig. 4b). When compared with zein 3L, the zein/PCL (20:10) 3L matrix exhibited a less controlled, more variable release, possibly due to the Tet being able to diffuse more freely through the more open, albeit hydrophobic, structure of the PCL-stabilised, fibrous matrix rather than it being physically entrapped within a dense film within the zein 3L matrix. Nevertheless, both 3L formulations exhibited the desired improvement in sustained release when compared to the single-layer equivalents as a result of the added drug diffusion barriers.

The Tet release profiles presented here are superior to those reported in the literature for zein-based electrospun scaffolds. While we have produced matrices capable of sustained drug release over a period >2 weeks, previously reported electrospun zein fibres have much shorter release windows. For example, crosslinked electrospun zein fibres released their encapsulated diclofenac within 10 h [35], while, using coaxial electrospinning techniques, ketoprofen [36] and ferulic acid [33] were encapsulated within zein fibres and subsequently released in 12 and 14 h, respectively. Blending zein and collagen has been shown to increase sustained delivery, with berberine largely plateauing by ~5 days [25]. Therefore, by using non-crosslinked electrospun zein as a single or 3L matrix, or by using a 3L zein/PCL (20:10) blend, longer-term delivery of therapeutic drugs is achievable. The timescales presented here are comparable with those of Tet released from zein/PLGA microparticles, which had been compressed into monolithic matrices [28].

However, the blended matrices described here exhibited a greater burst release than pure zein and, subsequently, a less controlled delivery of the encapsulated Tet. While this is not the ideal scenario, sustained release was still achieved and the increase in stability conferred on the matrix by the inclusion of PCL is significant. For translational applications, such as wound dressings, shrinkage is not desirable, as it would result in exposure of the wound. Blending zein with PCL successfully prevented this phenomenon.

Antibacterial efficacy of zein and zein/PCL matrices

The antibacterial activity of the Tet released from the zein-based electrospun matrices was assessed by bacteria inhibition experiments against the clinically-relevant meticillin-resistant *S. aureus* strain, MRSA252. Bacteria were challenged with discs of the electrospun matrices containing Tet (270 µg) and compared to a filter paper disc loaded with that same dose of Tet as a positive control. On a daily basis, the inhibition zone around each disc was measured and the discs transferred to a new agar plate. This process was continued for five days and the results are shown in Figure 5. All formulations exhibited a significant inhibition of *S. aureus* growth after 1 day, but all electrospun matrices had smaller zones of inhibition than the positive control, indicating a slower and more controlled release of Tet. This difference was statistically significant ($P<0.05$) for single-layer and 3L zein matrices and zein/PCL (20:10) 3L, but not for single-layer zein/PCL (20:10), correlating with the release profiles of these matrices, where zein/PCL (20:10) exhibited the highest burst release of these four formulations. Electrospun zein with no encapsulated Tet was ineffective, with no inhibition of bacterial growth (not shown), as previously demonstrated by Torres-Giner et al [34,53]. We have previously shown that electrospun PCL is also ineffective in this assay [13], suggesting that the antibacterial effects of the matrices in this study were solely due to the release of encapsulated Tet.

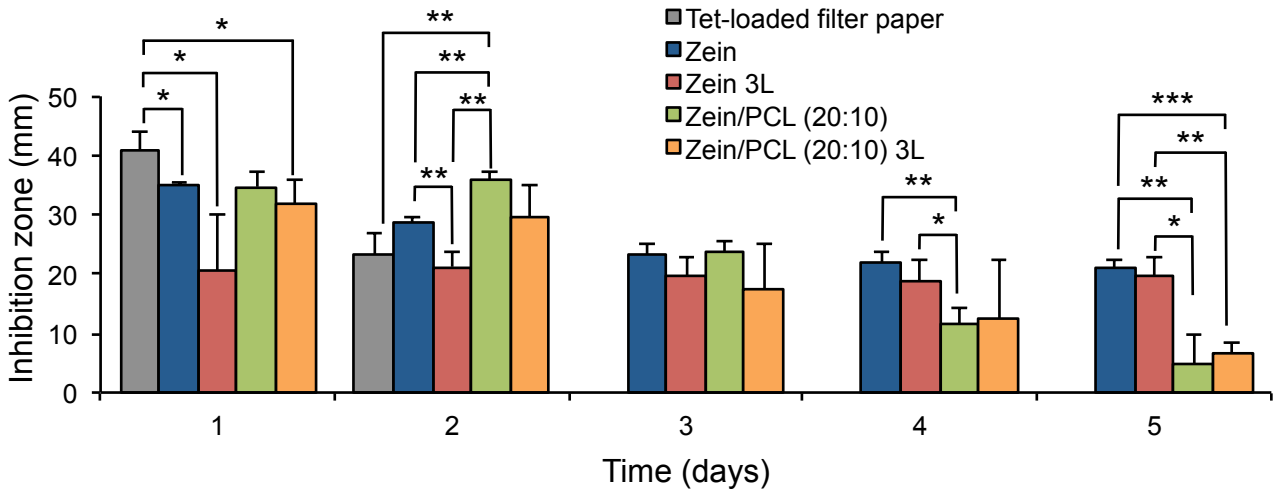

Fig. 5 Inhibition zones surrounding discs of Tet-containing electrospun zein and zein/PCL matrices in the *S. aureus* susceptibility test. The *error bars* represent the standard deviation of the mean (n=3 in triplicate; * $P<0.05$, ** $P<0.01$, *** $P<0.001$)

After 2 days, the inhibition zone around the positive control was significantly reduced, while those around the electrospun matrices were of a similar diameter to the 1day results, indicating a degree of controlled Tet release. The zein/PCL (20:10) matrix exhibited the largest inhibition zone, significantly greater than zein, zein 3L and the positive control (P<0.01), likely due to this formulation having the greatest rate of Tet release (i.e. burst) over a 48 h period (Fig. 4b). After 3 days, the positive control was spent and there was no evidence that the Tet-soaked filter paper possessed any additional antibacterial activity. However, all of the electrospun zein-based matrices continued to release a significant amount of Tet as evidenced by the substantial inhibition zones where the MRSA252 bacteria were unable to grow (Fig. 5). By day 4, all matrices were still able to kill the bacteria, although the inhibition zones surrounding the zein/PCL blends smaller than those of zein and zein 3L, with zein/PCL (20:10) being significantly less effective than the zein-only matrices (P<0.01 and P<0.05, respectively). This difference in efficacy was even greater at day 5, with zein and zein 3L maintaining similar zones of inhibition to those on previous days, while the zones of inhibition around the zein/PCL blends were further reduced. These differences correlate with the release profiles, with zein and zein 3L exhibiting higher release rates for a longer time period than the zein/PCL blends, where the Tet release rate dropped after the initial 2 days. However, despite the efficacy of the blended matrices being lower, all four formulations produced promising antibacterial activity over the whole 5 day period, demonstrating a sustained release of a biologically active amount of Tet, capable of killing and preventing the growth of a clinically-relevant strain of *S. aureus*.

We have demonstrated that electrospun zein matrices are unstable in an aqueous environment, even on an agar plate, with matrix shrinkage and deformation due to plasticization and film formation. However, blending zein with PCL (20:10 or 25:5 w/w) stabilized the electrospun fibres and prevented the matrices from shrinking. Such zein and zein/PCL matrices released Tet in a controlled manner, with a more extended release from 3L matrices. The Tet released from these matrices prevented the growth of MRSA252 for at least five days, supporting our proposal that these electrospun mats could be used as wound dressings to treat or prevent bacterial infection. Thus, we report the first demonstrated controlled delivery of a clinically-used antibiotic from electrospun triple-layer zein-based matrices. Indeed, the Tet release profiles presented here are superior to those reported in the literature for zein-based electrospun scaffolds which we consider to be due to the outer, drug-free layers acting as a diffusion barrier. We have designed and prepared a sustained release

formulation capable of killing *S. aureus* MRSA252, a multidrug-resistant clinical isolate that is a representative of an epidemic lineage endemic in UK hospitals.

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Conflict of Interest All three authors Nour Alhusein, Ian S. Blagbrough, and Paul A. De Bank declare that they have no conflict of interest. There were no experiments on human or animal subjects.

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